

(12)

EUROPEAN PATENT APPLICATION

(21) Application number: 83306507.1

(51) Int. Cl.²: C 12 N 15/00

C 07 H 21/04, C 12 P 21/00

//C12R1/645

(22) Date of filing: 26.10.83

(30) Priority: 12.01.83 US 457325
12.08.83 US 522909

(43) Date of publication of application:
22.08.84 Bulletin 84/34

(84) Designated Contracting States:
AT BE CH DE FR GB IT LI LU NL SE

(71) Applicant: CHIRON CORPORATION
4560 Horton Street
Emeryville California 94608(US)

(72) Inventor: Brake, Anthony John
2339 Hilgard Avenue
Berkeley California(US)

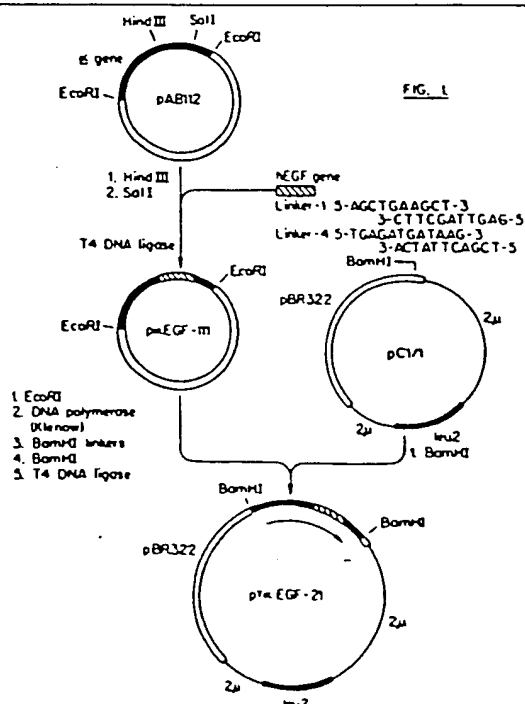
(74) Representative: Glawe, Defts, Moll & Partner
Patentanwälte
Rothenbaumchaussee 58 Postfach 2570
D-2000 Hamburg 13(DE)

(54) Secretory expression in eukaryotes.

(57) Methods and compositions are provided for producing polypeptide sequences in high yield by employing DNA constructs, wherein the DNA sequence encoding for the polypeptide of interest is preceded by a leader sequence and processing sequence for secreting and processing said polypeptide. In this manner, the mature polypeptide of interest may be isolated from the nutrient medium substantially free of major amounts of other proteins and cellular debris.

The yeast strain *S. cerevisiae* AB103 (pYEGF8) was deposited on January 5, 1983, at the A.T.C.C. and given accession No. 20658.

The plasmid pY_αEGF23 (pAB114-pC1/1) was deposited at the A.T.C.C. on August 12, 1983, and given Accession No. 40079.



EP 0 116 201 A1

9729-1-1/CCCCC2

SECRETORY EXPRESSION IN EUKARYOTES

BACKGROUND OF THE INVENTIONField of the Invention

5 Hybrid DNA technology has revolutionized the ability to produce polypeptides of an infinite variety of compositions. Since living forms are composed of proteins and employ proteins for regulation, the ability to duplicate these proteins at will offers
10 unique opportunities for investigating the manner in which these proteins function and the use of such proteins, fragments of such proteins, or analogs in therapy and diagnosis.

 There have been numerous advances in improv-
15 ing the rate and amount of protein produced by a cell. Most of these advances have been associated with higher copy numbers, more efficient promoters, and means for reducing the amount of degradation of the desired product. It is evident that it would be extremely
20 desirable to be able to secrete polypeptides of interest, where such polypeptides are the product of interest.

 Furthermore, in many situations, the polypeptide of interest does not have an initial methionine amino acid. This is usually a result of there being a
25 processing signal in the gene encoding for the polypeptide of interest, which the gene source recognizes and cleaves with an appropriate peptidase. Since in most situations, genes of interest are heterologous to the host in which the gene is to be expressed, such processing occurs imprecisely and in low yield in the expres-
30 sion host. In this case, while the protein which is obtained will be identical to the peptide of interest for almost all of its sequence, it will differ at the N-terminus which can deleteriously affect physiological
35 activity.

There are, therefore, many reasons why it would be extremely advantageous to prepare DNA sequences, which would encode for the secretion and maturing of the polypeptide product. Furthermore, where sequences can be found for processing, which result in the removal of amino acids superfluous to the polypeptide of interest, the opportunity exists for having a plurality of DNA sequences, either the same or different, in tandem, which may be encoded on a single transcript.

Description of the Prior Art

U.S. Patent No. 4,336,336 describes for prokaryotes the use of a leader sequence coding for a non-cytoplasmic protein normally transported to or beyond the cell surface, resulting in transfer of the fused protein to the periplasmic space. U.S. Patent No. 4,338,397 describes for prokaryotes using a leader sequence which provides for secretion with cleavage of the leader sequence from the polypeptide sequence of interest. U.S. Patent No. 4,338,397, columns 3 and 4, provide for useful definitions, which definitions are incorporated herein by reference.

Kurjan and Herskowitz, Cell (1982) 30:933-943 describes a putative α -factor precursor containing four tandem copies of mature α -factor, describing the sequence and postulating a processing mechanism. Kurjan and Herskowitz, Abstracts of Papers presented at the 1981 Cold Spring Harbor meeting on The Molecular Biology of Yeasts, page 242, in an Abstract entitled, "A Putative α -Factor Precursor Containing Four Tandem Repeats of Mature α -Factor," describe the sequence encoding for the α -factor and spacers between two of such sequences. Blair et al., Abstracts of Papers, ibid, page 243, in an Abstract entitled "Synthesis and Processing of Yeast Pheromones: Identification and Characterization of Mutants That Produce Altered α -

Factors," describe the effect of various mutants on the production of mature α -factor.

SUMMARY OF THE INVENTION

Methods and compositions are provided for
5 producing mature polypeptides. DNA constructs are provided which join the DNA fragments encoding for a yeast leader sequence and processing signal to heterologous genes for secretion and maturation of the polypeptide product. The construct of the DNA encoding for
10 the N-terminal cleavable oligopeptide and the DNA sequence encoding for the mature polypeptide product can be joined to appropriate vectors for introduction into yeast or other cell which recognizes the processing
signals for production of the desired polypeptide.
15 Other capabilities may also be introduced into the construct for various purposes.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a flow diagram indicating the construction of pY α EGF-21.

20 Fig. 2 shows sequences at fusions of hEGF to the vector. a. through e. show the sequences at the N-terminal region of hEGF, which differ among several constructions and f. shows the C-terminal region of hEGF.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

25 In accordance with the subject invention, eukaryotic hosts, particularly yeast are employed for the production of mature polypeptides, where such polypeptides may be harvested from a nutrient medium.
30 The polypeptides are produced by employing a DNA construct encoding for yeast leader and processing signals joined to a polypeptide of interest, which may be a single polypeptide or a plurality of polypeptides separated by processing signals. The resulting

construct encodes for a pre-pro-polypeptide which will contain the signals for secretion of the pre-pro-polypeptide and processing of the polypeptide, either intracellularly or extracellularly to the mature polypeptide.

The constructs of the subject invention will for example have at least the following formula defining a pro-polypeptide:

$$((R)_r-(GAXYCX)_n-Gene^*)_y$$

wherein:

R is CGX or AZZ, the codons coding for lysine and arginine, each of the Rs being the same or different;

r is an integer of from 2 to 4, usually 2 to 3, preferably 2 or 4;

X is any of the four nucleotides, T, G, C, or A;

Y is G or C;

y is an integer of at least one and usually not more than 10, more usually not more than four, providing for monomers and multimers;

Z is A or G; and

Gene* is a gene other than α -factor, usually foreign to a yeast host, usually a heterologous gene, desirably a plant or mammalian gene;

n is 0 or an integer which will generally vary from 1 to 4, usually 2 to 3.

The pro-polypeptide has an N-terminal processing signal for peptidase removal of the amino acids preceding the amino acids encoded for by Gene*.

For the most part, the constructs of the subject invention will have at least the following formula:

$$L-(R-S-(GAXYCX)_n)-Gene^*)_y$$

defining a pre-pro-polypeptide, wherein all the symbols except L and S have been defined, S having the same definition as R, there being 1R and 1S, and L is a leader sequence providing for secretion of the pre-pro-polypeptide. While it is feasible to have more

Rs and Ss there will usually be no advantage in the additional amino acids. Any leader sequence may be employed which provides for secretion, leader sequences generally being of about 30 to 120 amino acids, usually about 30 to 100 amino acids, having a hydrophobic region and having a methionine at its N-terminus.

The construct when n is 0 will have the following formula:

$$L-((R)_{r'}-Gene^*)_y$$

defining a pre-pro-polypeptide, wherein all the symbols have been defined previously, except r' , wherein:

r' is 2 to 4, preferably 2 or 4.

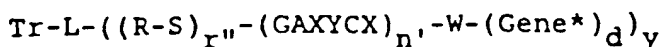
Of particular interest is the leader sequence of α -factor which is described in Kurjan and Herskowitz, supra, on page 937 or fragments or analogs thereof, which provide for efficient secretion of the desired polypeptides. Furthermore, the DNA sequence indicated in the article, which sequence is incorporated herein by reference, is not essential, any sequence which encodes for the desired oligopeptide being sufficient. Different sequences will be more or less efficiently translated.

While the above formulas are preferred, it should be understood, that with suppressor mutants, other sequences could be provided which would result in the desired function. Normally, suppressor mutants are not as efficient for expression and, therefore, the above indicated sequence or equivalent sequence encoding for the same amino acid sequence is preferred. To the extent that a mutant will express from a different codon the same amino acids which are expressed by the above sequence, then such alternative sequence could be permitted.

The dipeptides which are encoded for by the sequence in the parenthesis will be an acidic amino acid, aspartic or glutamic, preferably glutamic,

followed by a neutral amino acid, alanine and proline, particularly alanine.

In providing for useful DNA sequences which can be used for cassettes for expression, the following
5 sequence can be conveniently employed:



wherein:

Tr intends a DNA sequence encoding for the transcriptional regulatory signals, particularly the
10 promoter and such other regulatory signals as operators, activators, cap signal, signals enhancing ribosomal binding, or other sequence involved with transcriptional or translational control. The Tr sequence will generally be at least about 100bp and not more than about 2000bp.
15 Particularly useful is employing the Tr sequence associated with the leader sequence L, so that a DNA fragment can be employed which includes the transcriptional and translational signal sequences associated with the leader sequence endogenous to the host.
20 Alternatively, one may employ other transcriptional and translational signals to provide for enhanced production of the expression product;

d is 0 or 1, being 1 when y is greater than 1;

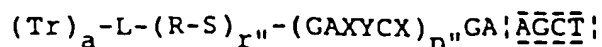
25 n' is a whole number, generally ranging from 0 to 3, more usually being 0 or 2 to 3;

r'' is 1 or 2;

W intends a terminal deoxyribosyl-3' group, or a DNA sequence which by itself or, when n' is other
30 than 0, in combination with the nucleotides to which it is joined, W defines a restriction site, having either a cohesive end or butt end, wherein W may have from 0 to about 20 nucleotides in the longest chain;

the remaining symbols having been defined
35 previously.

Of particular interest is the following construct:



wherein:

all of the symbols previously defined have the same definition;

5 a is 0 or 1 intending that the construct may or may not have the transcriptional and translational signals;

the nucleotides indicated in the broken box are intended not to be present but to be capable of
10 addition by adding an HindIII cleaved terminus to provide for the recreation of the sequence encoding for a dipeptide; and

n" will be 0 to 2, where at least one of the Xs and Ys defines a nucleotide, so that the sequence in
15 the parenthesis is other than the sequence GAAGCT.

The coding sequence of Gene* may be joined to the terminal T, providing that the coding sequence is in frame with the initiation codon and upon processing the first amino acid will be the correct amino acid for
20 the mature polypeptide.

The 3'-terminus of Gene* can be manipulated much more easily and, therefore, it is desirable to provide a construct which allows for insertion of Gene* into a unique restriction site in the construct. Such
25 a construct would provide for a restriction site with insertion of the Gene* into the restriction site to be in frame with the initiation codon. Such a construction can be symbolized as follows:



30 wherein:

those symbols previously defined have the same definition;

SC are stop codons;

Te is a termination sequence balanced with
35 the promoter Tr, and may include other signals, e.g. polyadenylation; and

b is an integer which will generally vary from about 0 to 4, more usually from 0 to 3, it being understood, that Gene* may include its own stop codons.

Illustrative of a sequence having the above
5 formula is where W is the sequence GA and n" is 2.

Of particular interest is where the sequence encoding the terminal dipeptide is taken together with W to define a linker or connector, which allows for recreation of the terminal sequence defining the
10 dipeptide of the processing signal and encodes for the initial amino acids of Gene*, so that the codons are in frame with the initiation codon of the leader. The linker provides for a staggered or butt ended termination, desirably defining a restriction site in conjunction with the successive sequences of the Gene*. Upon
15 ligation of the linker with Gene*, the codons of Gene* will be in frame with the initiation codon of the leader. In this manner, one can employ a synthetic sequence which may be joined to a restriction site in
20 the processing signal sequence to recreate the processing signal, while providing the initial bases of the Gene* encoding for the N-terminal amino acids. By employing a synthetic sequence, the synthetic linker can be a tailored connector having a convenient restriction site near the 3'-terminus and the synthetic
25 connector will then provide for the necessary codons for the 5'-terminus of the gene.

Alternatively, one could introduce a restriction endonuclease recognition site downstream from the
30 processing signal to allow for cleavage and removal of superfluous bases to provide for ligation of the Gene* to the processing signal in frame with the initiation codon. Thus the first codon would encode for the N-terminal amino acid of the polypeptide. Where T is
35 the first base of Gene*, one could introduce a restriction site where the recognition sequence is downstream from the cleavage site. For example, a Sau3A recogni-

tion sequence could be introduced immediately after the processing signal, which would allow for cleavage and linking of the Gene* with its initial codon in frame with the leader initiation codon. With restriction
5 endonucleases which have the recognition sequence distal and downstream from the cleavage site e.g. HqaI, we could define such sequence which could include a portion of the processing signal sequences. Other constructions can also be employed, employing such
10 techniques as primer repair and in vitro mutagenesis to provide for the convenient insertion of Gene* into the construct by introducing an appropriate restriction site.

The construct provides a portable sequence
15 for insertion into vectors, which provide the desired replication system. As already indicated, in some instances, it may be desirable to replace the wild type promoter associated with the leader sequence with a different promoter. In yeast, promoters involved with
20 enzymes in the glycolytic pathway can provide for high rates of transcription. These promoters are associated with such enzymes as phosphoglucosomerase, phosphofructokinase, phosphotriose isomerase, phosphoglucomutase, enolase, pyruvic kinase, glyceraldehyde-3-phosphate dehydrogenase, and alcohol dehydrogenase.
25 These promoters may be inserted upstream from the leader sequence. The 5'-flanking region to the leader sequence may be retained or replaced with the 3'-sequence of the alternative promoter. Vectors can be
30 prepared and have been reported which include promoters having convenient restriction sites downstream from the promoter for insertion of such constructs as described above.

The final construct will be an episomal
35 element capable of stable maintenance in a host, particularly a fungal host such as yeast. The construct will include one or more replication systems, desirably

two replication systems, allowing for maintenance in the expression host and cloning in a prokaryote. In addition, one or more markers for selection will be included, which will allow for selective pressure for maintenance of the episomal element in the host. Furthermore, the episomal element may be a high or low copy number, the copy number generally ranging from about 1 to 200. With high copy number episomal elements, there will generally be at least 10, preferably at least 20, and usually not exceeding about 150, more usually not exceeding about 100 copy number. Depending upon the Gene*, either high or low copy numbers may be desirable, depending upon the effect of the episomal element on the host. Where the presence of the expression product of the episomal element may have a deleterious effect on the viability of the host, a low copy number may be indicated.

Various hosts may be employed, particularly mutants having desired properties. It should be appreciated that depending upon the rate of production of the expression product of the construct, the processing enzyme may or may not be adequate for processing at that level of production. Therefore, a mutant having enhanced production of the processing enzyme may be indicated or enhanced production of the enzyme may be provided by means of an episomal element. Generally, the production of the enzyme should be of a lower order than the production of the desired expression product.

Where one is using α -factor for secretion and processing, it would be appropriate to provide for enhanced production of the processing enzyme Dipeptidyl Amino Peptidase A, which appears to be the expression product of STE13. This enzyme appears to be specific for X-Ala- and X-Pro-sequences, where X in this instance intends an amino acid, particularly, the dicarboxylic acid amino acids.

Alternatively, there may be situations where intracellular processing is not desired. In this situation, it would be useful to have a stel3 mutant, where secretion occurs, but the product is not processed. In this manner, the product may be subsequently processed in vitro.

Host mutants which provide for controlled regulation of expression may be employed to advantage. For example, with the constructions of the subject invention where a fused protein is expressed, the transformants have slow growth which appears to be a result of toxicity of the fused protein. Thus, by inhibiting expression during growth, the host may be grown to high density before changing the conditions to permissive conditions for expression.

A temperature-sensitive sir mutant may be employed to achieve regulated expression. Mutation in any of the SIR genes results in a non-mating phenotype due to in situ expression of the normally silent MATa and MAT α sequences present at the HML and HMR loci.

Furthermore, as already indicated, the Gene* may have a plurality of sequences in tandem, either the same or different sequences, with intervening processing signals. In this manner, the product may be processed in whole or in part, with the result that one will obtain the various sequences either by themselves or in tandem for subsequent processing. In many situations, it may be desirable to provide for different sequences, where each of the sequences is a subunit of a particular protein product.

The Gene* may encode for any type of polypeptide of interest. The polypeptide may be as small as an oligopeptide of 8 amino acids or may be 100,000 daltons or higher. Usually, single chains will be less than about 300,000 daltons, more usually less than about 150,000 daltons. Of particular interest are polypeptides of from about 5,000 to 150,000 daltons,

more particularly of about 5,000 to 100,000 daltons. Illustrative polypeptides of interest include hormones and factors, such as growth hormone, somatomedins epidermal growth factor, the endocrine secretions, such as luteinizing hormone, thyroid stimulating hormone, oxytocin, insulin, vasopressin, renin, calcitonin, follicle stimulating hormone, prolactin, etc.; hematopoietic factors, e.g. erythropoietin, colony stimulating factor, etc.; lymphokines; globins; globulins, e.g. immunoglobulins; albumins; interferons, such as α , β and γ ; repressors; enzymes; endorphins e.g. β -endorphin, enkephalin, dynorphin, etc.

Having prepared the episomal elements containing the constructs of this invention, one may then introduce such element into an appropriate host. The manner of introduction is conventional, there being a wide variety of ways to introduce DNA into a host. Conveniently, spheroplasts are prepared employing the procedure of, for example, Hinnen et al., PNAS USA (1978) 75:1919-1933 or Stinchcomb et al., EP 0 045 573 A2. The transformants may then be grown in an appropriate nutrient medium and where appropriate, maintaining selective pressure on the transformants. Where expression is inducible, one can allow for growth of the yeast to high density and then induce expression. In those situations, where a substantial proportion of the product may be retained in the periplasmic space, one can release the product by treating the yeast cells with an enzyme such as zymolase or lyticase.

The product may be harvested by any convenient means, purifying the protein by chromatography, electrophoresis, dialysis, solvent-solvent extraction, etc.

In accordance with the subject invention, one can provide for secretion of a wide variety of polypeptides, so as to greatly enhance product yield, simplify purification, minimize degradation of the desired

product, and simplify processing, equipment, and engineering requirements. Furthermore, utilization of nutrients based on productivity can be greatly enhanced, so that more economical and more efficient production of polypeptides may be achieved. Also, the use of yeast has many advantages in avoiding enterotoxins, which may be present with prokaryotes, and employing known techniques, which have been developed for yeast over long periods of time, which techniques include isolation of yeast products.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

A synthetic sequence for human epidermal growth factor (EGF) based on the amino acid sequence of EGF reported by H. Gregory and B.M. Preston Int. J. Peptide Protein Res. 9, 107-118 (1977) was prepared, which had the following sequence.

5' AACTCCGACTCCGAATGTCCATTGTCCCACGACGGTTACTGTTGCACGACGGTGTGTTG
3' TTGAGGCTGAGGCTTACAGGTAACAGGGTGCTGCCAATGACAAACGTGCTGCCACAAACA

ATGTACATCGAAGCTTTGGACAAGTACGCTTGTAAGTGTGTTGTTGGTTACATCGGTGAA
TACATGTAGCTTCGAAACCTGTTTCATGCGAACATTGACACAACAACCAATGTAGCCACTT

AGATGTCAATACAGAGACTTGAAGTGGTGGGAATTGAGATGA
TCTACAGTTATGTCTCTGAACTTCACCACCCTTAAGTCTACT,

where 5' indicates the promoter proximal end of the sequence. The sequence was inserted into the EcoRI site of pBR328 to produce a plasmid p328EGF-1 and cloned.

Approximately 30µg of p328EGF-1 was digested with EcoRI and approximately 1µg of the expected 190 base pair EcoRI fragment was isolated. This was followed by digestion with the restriction enzyme HgaI.

Two synthetic oligonucleotide connectors HindIII-HgaI and HgaI-SalI were then ligated to the 159 base pair HgaI fragment. The HgaI-HindIII linker had the following sequence:

5 AGCTGAAGCT
CTTCGATTGAG

This linker restores the α -factor processing signals interrupted by the HindIII digestion and joins the HgaI end at the 5'-end of the EGF gene to the HindIII end of pAB112.

The HgaI-SalI linker had the following sequence:

15 TGAGATGATAAG
ACTATTTCAGCT
This linker has two stop codons and joins the HgaI end at the 3'-end of the EGF gene to the SalI end of pAB112.

The resulting 181 base pair fragment was purified by preparative gel electrophoresis and ligated to 100ng of pAB112 which had been previously completely digested with the enzymes HindIII and SalI. Surprisingly, a deletion occurred where the codon for the 3rd and 4th amino acids of EGF, asp and ser, were deleted, with the remainder of the EGF being retained.

25 pAB112 is a plasmid containing a 1.75kb EcoRI fragment with the yeast α -factor gene cloned in the EcoRI site of pBR322 in which the HindIII and SalI sites had been deleted (pAB11). pAB112 was derived from plasmid pAB101 which contains the yeast α -factor gene as a partial Sau3A fragment cloned in the BamHI site of plasmid YEp24. pAB101 was obtained by screening a yeast genomic library in YEp24 using a synthetic 20-mer oligonucleotide probe (3'-GGCCGGTTGGTTACATGATT-5') homologous to the published α -factor coding region (Kurjan and Herskowitz, Abstracts 1981 Cold Spring Harbor meeting on the Molecular Biology of Yeasts, page 242).

The resulting mixture was used to transform E. coli HB101 cells and plasmid pAB201 obtained.

Plasmid pAB201 (5µg) was digested to completion with the enzyme EcoRI and the resulting fragments were:

- 5 a) filled in with DNA polymerase I Klenow fragment;
- b) ligated to an excess of BamHI linkers; and
- c) digested with BamHI. The 1.75kbp EcoRI fragment was isolated by preparative gel electrophoresis and approximately 100ng of the fragment was ligated to
- 10 100ng of pCl/1, which had been previously digested to completion with the restriction enzyme BamHI and treated with alkaline phosphatase.

- Plasmid pCl/1 is a derivative of pJDB219, Beggs, Nature (1978) 275:104, in which the region
- 15 corresponding to bacterial plasmid pMB9 in pJDB219 has been replaced by pBR322 in pCl/1. This mixture was used to transform E. coli HB101 cells. Transformants were selected by ampicillin resistance and their plasmids analyzed by restriction endonucleases. DNA
 - 20 from one selected clone (pYEGF-8) was prepared and used to transform yeast AB103 cells. Transformants were selected by their leu⁺ phenotype.

- Fifty milliliter cultures of yeast strain AB103 (α, pep 4-3, leu 2-3, leu 2-112, ura 3-52, his
- 25 4-580) transformed with plasmid pYEGF-8 (deposited at the American Type Culture Collection on 5th January 1983 and given ATCC Accession no. 20658) were grown at 30° in -leu medium to saturation (optical density at 600nm of 5) and left shaking at 30° for an additional
 - 30 12 hr period. Cell supernatants were collected by centrifugation and analyzed for the presence of human EGF using the fibroblast receptor competition binding assay. The assay of EGF is based on the ability of both mouse and human EGF to compete with ¹²⁵I-labeled
 - 35 mouse EGF for binding sites on human foreskin fibroblasts. Standard curves can be obtained by measuring the effects of increasing quantities of EGF on the binding of a standard amount of ¹²⁵I-labeled mouse EGF. Under these conditions 2 to 20 ng of EGF are readily

measurable. Details on the binding of ^{125}I -labeled epidermal growth factor to human fibroblasts have been described by Carpenter et al., J. Biol. Chem. 250, 4297 (1975). Using this assay it is found that the culture medium contains 71mg of human EGF per liter.

For further characterization, human EGF present in the supernatant was purified by absorption to the ion-exchange resin Biorex-70 and elution with HCl 10mM in 80% ethanol. After evaporation of the HCl and ethanol the EGF was solubilized in water. This material migrates as a single major protein of MW approx. 6,000 in 17.5% SDS gels, roughly the same as authentic mouse EGF (MW~6,000). This indicates that the α -factor leader sequence has been properly excised during the secretion process. Analysis by high resolution liquid chromatography (microbondapak C18, Waters column) indicates that the product migrates with a retention time similar to an authentic mouse EGF standard. However, protein sequencing by Edman degradation showed that the N-terminus retained the glu-ala sequence.

A number of other constructions were prepared using different constructions for joining hEGF to the α -factor secretory leader sequence, providing for different processing signals and site mutagenesis. In Fig. 2 a. through e. show the sequence of the fusions at the N-terminal region of hEGF, which sequence differ among several constructions. f. shows the sequences at the C-terminal region of hEGF, which is the same for all constructions. Synthetic oligonucleotide linkers used in these constructions are boxed.

These fusions were made as follows. Construction (a) was made as described above. Construction (b) was made in a similar way except that linker 2 was used instead of linker 1. Linker 2 modifies the α -factor processing signal by inserting an additional processing

site (ser-leu-asp-lys-arg) immediately preceding the hEGF gene. The resulting yeast plasmid is named pYoEGF-22. Construction (c), in which the dipeptidyl aminopeptidase maturation site (glu-ala) has been removed, was obtained by in vitro mutagenesis of construction (a). A PstI-SalI fragment containing the α -factor leader-hEGF fusion was cloned in phage M13 and isolated in a single-stranded form. A synthetic 31-mer of sequence 5'-TCTTTGGATAAAAGAACTCCGACTCCCG-3' was synthesized and 70 picomoles were used as a primer for the synthesis of the second strand from 1 picomole of the above template by the Klenow fragment of DNA polymerase. After fill-in and ligation at 14° for 18 hrs, the mixture was treated with S_1 nuclease (5 units for 15 min) and used to transfect E. coli JM101 cells. Bacteriophage containing DNA sequences in which the region coding for (glu-ala) was removed were located by filter plaque hybridization using the 32 P-labeled primer as probe. RF DNA from positive plaques was isolated, digested with PstI and SalI and the resulting fragment inserted in pAB114 which had been previously digested to completion with SalI and partially with PstI and treated with alkaline phosphatase.

The plasmid pAB114 was derived as follows: plasmid pAB112 was digested to completion with HindIII and then religated at low (4 μ g/ml) DNA concentration and plasmid pAB113 was obtained in which three 63bp HindIII fragments have been deleted from the α -factor structural gene, leaving only a single copy of mature α -factor coding region. A BamHI site was added to plasmid pAB11 by cleavage with EcoRI, filling in of the overhanging ends by the Klenow fragment of DNA polymerase, ligation of BamHI linkers, cleavage with BamHI and religation to obtain pAB12. Plasmid pAB113 was digested with EcoRI, the overhanging ends filled in, and ligated to BamHI linkers. After digestion with BamHI the 1500bp fragment was gel-purified and ligated

to pAB12 which had been digested with BamHI and treated with alkaline phosphatase. Plasmid pAB114, which contains a 1500bp BamHI fragment carrying the α -factor gene, was obtained. The resulting plasmid (pAB114
5 containing the above described construct) is then digested with BamHI and ligated into plasmid pC1/1.

The resulting yeast plasmid is named pY α EGF-23 and was deposited at the American Type Culture Collection on 12th August 1983 under ATCC Accession no. 40079.

10 Construction (d), in which a new KpnI site was generated, was made as described for construction (c) except that the 36-mer oligonucleotide primer of sequence 5'-GGGTACCTTTGGATAAAAGAACTCCGACTCCGAAT-3' was used. The resulting yeast plasmid is named pY α EGF-24.

15 Construction (e) was derived by digestion of the plasmid containing construction (d) with KpnI and SalI instead of linker 1 and 2. The resulting yeast plasmid is named pY α EGF-25.

Yeast cells transformed with pY α EGF-22 were
20 grown in 15 ml cultures. At the indicated densities or times, cultures were centrifuged and the supernatants saved and kept on ice. The cell pellets were washed in lysis buffer (0.1 Triton X-100, 10mM NaHPO₄ pH 7.5) and broken by vortexing (5min in 1min intervals with
25 cooling on ice in between) in one volume of lysis buffer and one volume of glass beads. After centrifugation, the supernatants were collected and kept on ice. The amount of hEGF in the culture medium and cell
30 binding competition assay. Standard curves were obtained by measuring the effects of increasing quantities of mouse EGF on the binding of a standard amount ¹²⁵I-labeled mouse EGF.

Proteins were concentrated from the culture
35 media by absorption on Bio-Rex 70 resin and elution with 0.01 HCl in 80% ethanol and purified by high performance liquid chromatography (HPLC) on a reverse phase C18 column. The column was eluted at a flow rate of 4ml/min with a linear gradient of 5% to 80% aceto-

nitrite containing 0.2% trifluoroacetic acid in 60min. Proteins (200-800 picomoles) were sequenced at the amino-terminal end by the Edman degradation method using a gas-phase protein sequencer Applied Biosystems model 470A. The normal PROTFA program was used for all the analyses. Dithiothreitol was added to S2 (ethyl acetate: 20mg/liter) and S3 (butyl chloride: 10mg/liter) immediately before use. All samples were treated with 1N HCl in methanol at 40° for 15min to convert PTH-aspartic acid and PTH-glutamic acid to their methyl esters. All PTH-amino acid identifications were performed by reference to retention times on a IBM CN HPLC column using a known mixture of PTH-amino acids as standards.

Secretion from pY α EGF-22 gave a 4:1 mole ratio of native N-terminus hEGF to glu-ala terminated hEGF, while secretion from pY α EGF-23-25 gave only native N-terminated hEGF. Yields of hEGF ranged from 5 to 8 μ g/ml measured either as protein or in a receptor binding assay.

The strain JRY188 (MAT sir3-8 leu2-3 leu2-112 trp1 ura3 his4 rme) was transformed with pY α EGF-21 and leucine prototrophs selected at 37°. Saturated cultures were then diluted 1/100 in fresh medium and grown in leucine selective medium at permissive (24°) and non-permissive (36°) temperatures and culture supernatants were assayed for the presence of hEGF as described above. The results are shown in the following table.

Regulated synthesis and secretion of hEGF in transformed yeast sir3 temperature-sensitive mutants.

5	Temperature	Transformant	O.D.650	hEGF(μg/ml)
	<hr/>			
	36°	3a	3.5	0.010
			5.4	0.026
		3b	3.6	0.020
10			6.4	0.024
	<hr/>			
	24°	3a	0.4	34
			1.3	145
			2.1	1075
15			4.0	3250
		3b	0.4	32
			1.4	210
			2.2	1935
20			4.2	4600

These results indicate that the hybrid α -factor/EGF gene is being expressed under mating type regulation, even though it is present on a high copy number plasmid.

25 In accordance with the subject invention, novel constructs are provided which may be inserted into vectors to provide for expression of polypeptides having an N-terminal leader sequence and one or more processing signals to provide for secretion of the

30 polypeptide as well as processing to result in a mature polypeptide product free of superfluous amino acids. Thus, one can obtain a polypeptide having the identical sequence to a naturally occurring polypeptide. In addition, because the polypeptide can be produced in

35 yeast, glycosylation can occur, so that products can be obtained which are identical to the naturally occurring

products. Furthermore, because the product is secreted, greatly enhanced yields can be obtained based on cell population and processing and purification are greatly simplified. In addition, employing mutant hosts,
5 expression can be regulated to be turned off or on, as desired.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it
10 will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

CLAIMS

1. A DNA construct encoding a pre-pro-polypeptide, said DNA construct encoding pre-pro-polypeptide comprising a yeast leader sequence, processing signals
5 for processing the pre-pro-polypeptide to a mature polypeptide and a gene encoding a polypeptide other than the wild type gene associated with said leader sequence.

2. A DNA construct according to Claim 1, including at the 5' end of the sequence a yeast promoter and wherein said gene is heterologous to said
10 yeast host.

3. A DNA construct according to Claim 2, wherein said yeast promoter is the σ -factor promoter and said yeast leader is a leader sequence encoding for
15 at least a major portion of the σ -factor leader and is capable of providing for secretion.

4. A DNA construct according to Claim 2, wherein said gene is a mammalian gene.

5. A DNA construct comprising a sequence of
20 the following formula:

$$L-((R)_r-(GAXYCX)_n-Gene^*)_y$$

wherein:

L is a leader sequence recognized by yeast for secretion;

25 R is a codon coding for arginine or lysine;

r is an integer of from 2 to 4;

X is any nucleotide;

Y is guanosine or cytosine;

y is an integer of from about 1 to 10;

30 Gene* is a gene foreign to yeast; and

n is 0 or 1 to 4.

6. A DNA construct according to Claim 5, wherein n is 0 to 4 and the nucleotides of said Gene* proximal to R at least in part define a recognition site for a restriction endonuclease.

5 7. A DNA construct according to Claim 6, wherein said leader sequence is the α -factor leader sequence.

8. A DNA construct according to Claim 7, wherein n is 0.

10 9. A DNA construct of the formula:
Tr-L-(R-S-(GAXYCX)_n-W-(Gene*)_d)_y

wherein:

Tr is a sequence having transcriptional and translational regulatory signals for initiation and
15 processing of transcription and translation, wherein said regulatory signals are recognized by yeast;

L is a leader sequence for secretion by yeast;

20 R and S are codons expressing arginine and lysine;

X is any nucleotide;

Y is cytosine or guanosine;

y is an integer of from 1 to 4;

n' is a whole number of from 0 to 4;

25 W is a deoxyribosyl-3' group or when n' is other than 0, one or more nucleotides which by themselves or together with the hexanucleotide in the parenthesis define a restriction site;

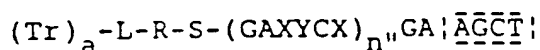
Gene* either by itself or taken together with
30 W defines a polypeptide sequence foreign to yeast; and
d is 0 or 1, being 1, when y is greater than 1.

10. A DNA construct according to Claim 9, wherein Tr is a sequence defining the regulatory signals for α -factor, d is 1 and Gene* and W are taken together to define a polypeptide foreign to yeast.

5 11. A DNA construct according to Claim 9 wherein n' is 0.

12. A DNA construct according to Claim 11, wherein said polypeptide product is a mammalian polypeptide.

10 13. A DNA construct comprising a sequence of the formula:



wherein:

15 Tr is a sequence defining transcriptional and translational regulatory signals for initiation and processing of transcription and translation recognized by yeast;

a is 0 or 1;

L is a leader sequence recognized by yeast;

20 R and S are codons encoding for lysine and arginine;

X is any nucleotide;

Y is cytosine or guanosine;

n'' is 2 to 4;

25 the nucleotides in the broken box indicate the nucleotides which are complementary to the overhang of the non-coding chain to define a HindIII restriction site.

14. A DNA construct according to Claim 13, where Tr is a sequence defining the transcriptional and translational regulatory signals of α -factor.

15. An expression episomal element comprising a replication system for providing stable maintenance in yeast and a sequence of the formula:

Tr-L-(R)_r-(GAXYCX)_n-W-Te

wherein:

Tr is a sequence defining transcriptional and translational regulatory signals for initiation and processing of transcription and translation in yeast;

L is a leader sequence recognized by yeast for secretion;

R is a codon defining arginine or lysine;

r' is a whole number in the range of 2 to 4;

X is any nucleotide;

Y is cytosine or guanosine;

n' is a whole number in the range of 0 to 4;

W is a nucleotide sequence of at least 1 nucleotide, which by itself or when n' is other than 0, in conjunction with nucleotides in the parenthesis defines a restriction site;

Te is a sequence defining a terminator balanced with said transcriptional initiator sequence.

16. An expression episomal element according to Claim 15 wherein Tr is derived from α -factor and n' is 2 to 3.

17. An expression episomal element according to Claim 14, wherein Tr is derived from α -factor and n' is 0.

18. An episomal expression vector according to Claim 17, having a gene foreign to yeast intermediate

R and Te and in reading frame with the initiation codon of L.

19. An episomal expression element according to Claim 18, wherein said gene is a mammalian gene.

5 20. An episomal element according to Claim 19, wherein said mammalian gene is human epidermal growth factor.

10 21. An episomal expression vector according to Claim 16, having a gene foreign to yeast intermediate the nucleotides in the parentheses and Te and in reading frame with the initiation codon of L.

15 22. A method for producing a polypeptide foreign to yeast and having such polypeptide secreted into the culture medium, said method comprising:
growing yeast containing an episomal expression elements according to Claim 16, whereby the encoding sequences are expressed to produce a pre-pro-polypeptide; and
said pre-pro-polypeptide is at least partially
20 processed and secreted.

23. An episomal expression vector according to Claim 17, having a gene foreign to yeast intermediate the nucleotides in the parentheses and Te and in reading frame with the initiation codon of L.

25 24. A method for producing a polypeptide foreign to yeast and having such polypeptide secreted into the culture medium, said method comprising:
growing yeast mutants containing an episomal expression element according to Claim 16, wherein said
30 mutant permits external regulation of expression,

whereby the encoding sequences are expressed to produce a pre-pro-polypeptide under permissive conditions; and said pre-pro-polypeptide is at least partially processed and secreted.

5 25. A method according to Claim 24, wherein said mutant yeast is a temperature-sensitive sir mutant.

10 26. A method for producing a polypeptide foreign to yeast and having such polypeptide secreted into the culture medium, said method comprising:
 growing yeast mutants containing an episomal expression element according to Claim 17, wherein said mutant permits external regulation of expression, whereby the encoding sequences are expressed to produce
15 a pre-pro-polypeptide under permissive conditions; and
 said pre-pro-polypeptide is at least partially processed and secreted.

20 27. A method according to Claim 26, wherein said mutant yeast is a temperature-sensitive sir mutant.

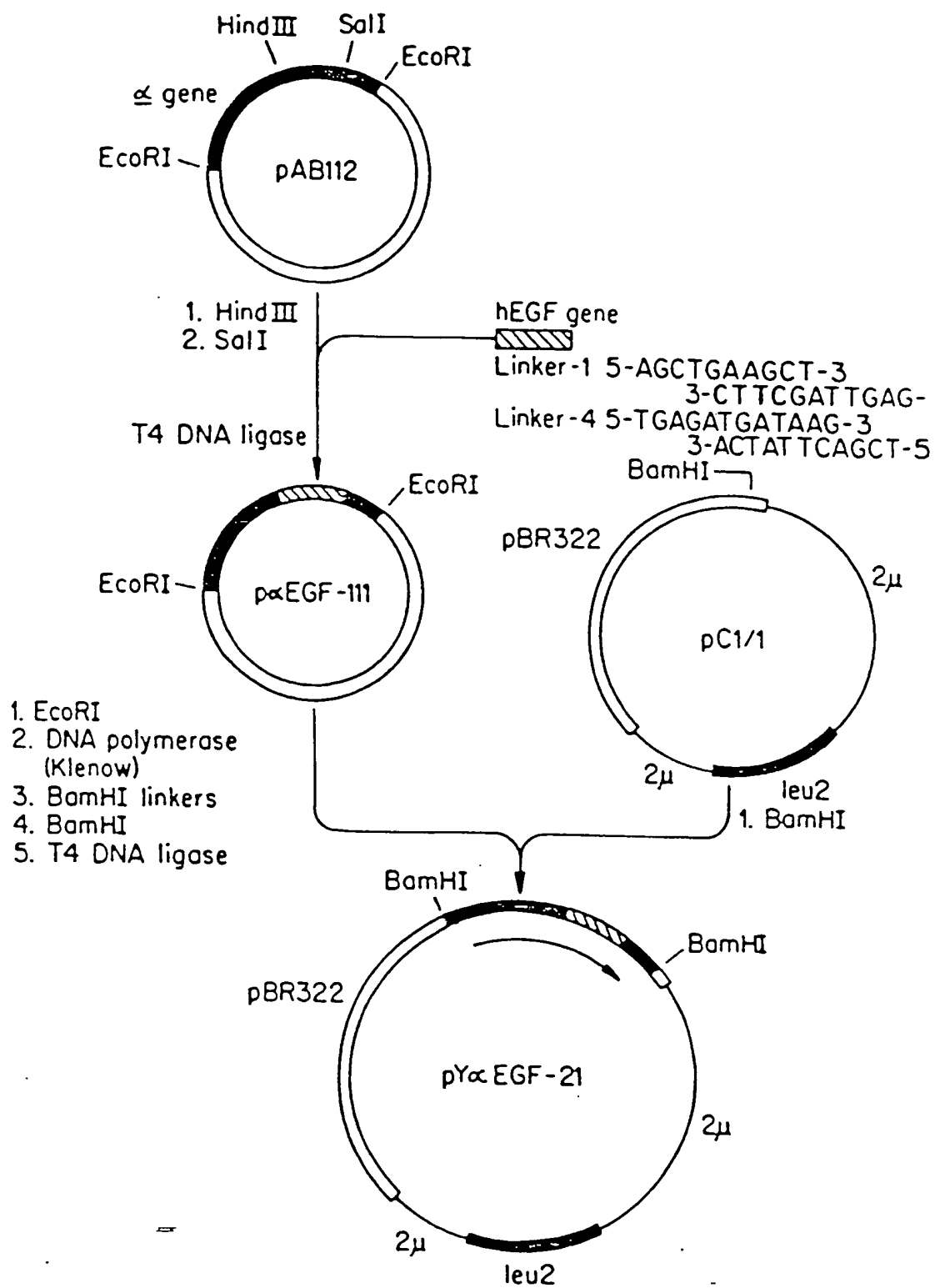


FIG. 1.

2/2

80 85 90 5
 Gly Val Ser Leu Asp Lys Arg Glu Ala Glu Ala Glu Asn Ser Asp Ser Glu
 a. GGG GTA TCT TTG GAT AAA AGA GAG GCT GAA GCT GAA GCT AAC TCC GAC TCC GAA
 CCC CAT AGA AAC CTA TTT TCT CTC CGA CTT CGA CTT CGA TTG AGG CTG AGG CTT.
 80 85 90 5
 Gly Val Ser Leu Asp Lys Arg Glu Ala Glu Ala Ser Leu Asp Lys Arg Asn Ser Asp Ser Glu
 b. GGG GTA TCT TTG GAT AAA AGA GAG GCT GAA GCT TCT TTG GAT AAA AGA AAC TCC GAC TCC GAA
 CCC CAT AGA AAC CTA TTT TCT CTC CGA CTT CGA AGA AAC CTA TTT TCT TTG AGG CTG AGG CTT
 80 85 5
 Gly Val Ser Leu Asp Lys Arg Asn Ser Asp Ser Glu
 c. GGG GTA TCT TTG GAT AAA AGA AAC TCC GAC TCC GAA
 CCC CAT AGA AAC CTA TTT TCT TTG AGG CTG AGG CTT
 80 85 5
 Gly Val Pro Leu Asp Lys Arg Asn Ser Asp Ser Glu
 d. GGG GTA CCT TTG GAT AAA AGA AAC TCC GAC TCC GAA
 CCC CAT GGA AAC CTA TTT TCT TTG AGG CTG AGG CTT
 80 85 5
 Gly Val Gin Leu Asp Lys Arg Asn Ser Asp Ser Glu
 e. GGG GTA GAG CTG GAT AAA AGA AAC TCC GAC TCC GAA
 CCC CAT GTC GAC CTA TTT TCT TTG AGG CTG AGG CTT
 50 53
 Trp Trp Glu Leu Arg
 Linker - 4
 TGG TGG GAA TTG AGA TGA TAA GTC GAC CGA TG
 ACC ACC CTT AAC TCT ACT ATT CAG CTG GCT AC
 Linker - 1
 Linker - 2
 Linker - 3
 (pY α EGF-21)
 (pY α EGF-22)
 (pY α EGF-23)
 (pY α EGF-24)
 (pY α EGF-25)

FIG. 2.



DOCUMENTS CONSIDERED TO BE RELEVANT			EP 83306507.1
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 7)
X,P	EP - A2 - 0 088 632 (GENENTECH) • Claims 1,5,15; page 5, lines 21-28; page 6, lines 4-19 • --	1,2,4, 22,24, 26	C 12 N 15/00 C 07 H 21/04 C 12 P 21/00 //C 12 R 1/645
A	EP - A1 - 0 060 057 (BOARD OF REGENTS UNIVERSITY OF WASHINGTON) • Claims 1,6,7 • --	1,2	
A	GB - A - 2 100 737 (CELLTECH) • Abstract; claim 4 • --	1	
A,P	EP - A2 - 0 073 635 (KINGSMAN) • Claim 1 • --	1,2	
A,P	GB - A - 2 104 902 (GENENTECH) • Claim 1 • --	1,2,4	TECHNICAL FIELDS SEARCHED (Int. Cl. 7)
A,D	CELL, vol. 30, no. 3, October 1982, Cambridge, Massachusetts and London J. KURJAN, I. HERSKOWITZ "Structure of a Yeast Pheromone Gene (MF α): A Putative α -Factor Precursor Contains Four Tandem Copies of Mature α -Factor" pages 933-943 • Page 933 (SUMMARY) • --	3	C 12 N C 12 P C 07 H
A,D	US - A - 4 338 397 (GILBERT) • Claims 1,7,8,12,16 • ----	1,2	
The present search report has been drawn up for all claims			
Place of search VIENNA		Date of completion of the search 13-04-1984	Examiner FARNIOK
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

☒ **BLACK BORDERS**

☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**

☐ **FADED TEXT OR DRAWING**

☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**

☐ **SKEWED/SLANTED IMAGES**

☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**

☐ **GRAY SCALE DOCUMENTS**

☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**

☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**

☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.